STUDY ON BIODEGRADATION OF MIRI AND MASILA CRUDE OIL AND USED CAR OIL BY MICROORGANISMS ISOLATED FROM MALAYSIAN SOIL AND THE EFFECT OF AERATION AND NPK ADDITION ON BIODEGRADATION PROCESS

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ABSTRACT

In this study, five contaminated soil samples with benzene, used car oil and diesel were collected from five car workshops in Kuala Lumpur area, Malaysia. The microbial strains were isolated using selective media (agar containing crude oil, used car oil and benzene). Microorganisms were identified by biochemical test and then used in biodegradation experiment of two types of crude oils (Miri and Masila) and used car oil. Qualitative determination of the degradation capacity of crude oils and used car oil was driven in 24 well cell culture cluster - flat bottom, adding to each well nutrient broth medium, crude oil or used car oil and the isolated microorganism cultured in the tubes (single and mixtures) and incubated at 27-°C for 30 days. For hydrocarbons rate of biodegradation measurement, sterilized soil was distributed in petri dishes, 3% w/w of two crude oils and used car oil were added, separately and then supplemented with isolated strains (single and mixtures). One Petri dish was used as a control without any microbial addition. Absorbance was determined by spectrophotometer at 360 nm and at 340 nm for crude oils and used car oil, respectively. The effect of aeration, added NPK and added microbial degraders on biodegradation of Masila crude oil and used car oil was studied. Soil was supplemented with 20% Masila crude oil and used car oil, separately. The soil was distributed into containers. Container (1) was aerated twice a week, NPK 1:1:1 was added to container (2), mixture of all isolated strains with degrading capacity was added to container (3). Finally, NPK with continued aeration in addition to the microbial mixture were applied to the last container (4). One container was used as a control without any addition (soil and contaminant only). Colony Forming Unit (CFU) of total heterotrophic microbes and hydrocarbon utilizing microbes, PH and percentage of oils degradation were determined. 22 microbial strains were isolated and identified as, *Achrombacter*, *Aeromonas*, *Klebsiella pneumonia*, *Pseudomonas*, *Corynebacterium*, *Penicillium*, *Enterobacteriaceae* (*Enteric rods*), *Actinobacillus*, *Streptomyces*, budding yeast cells, *Cladosporum* and *Geotrichum* spp. The highest biodegradation result in Miri crude oil after 30 days were 54.33% and 84.61% for strain Z13 (*Corynebacterium* spp.) and microbial mixture of the strains isolated from Rawang and Serdang area (MS), respectively. While in Masila crude oil was 33.81% for Strain A3 (*Klebsiella pneumonia*) and 49.47% for microbial mixture of the strains isolated from Serdang (Smix). In used car oil biodegradation experiment, strain Z4 (*Corynebacterium*) had the highest degradation with 72.9%. While microbial mixture of the strains isolated from Kajang (Zmix) had 72.4 % of degradation. In the experiment of the effect of aeration, added NPK and added microbial degraders on biodegradation, the aerated container showed 56.62% of degradation after 42 days in Masila crude oil, while the container which contained NPK and was aerated and supplemented with isolated strains showed 66% degradation in used car oil. Generally, two bacterial species and one fungal species isolated were found to be effective degraders (*Corynebacterium* spp, *Streptomyces* spp. and *Cladosporum* spp,), respectively. The highest degradability by single strains was on used car oil which might be due to the adaptability of the isolated microbes to use it. The microbial mixtures showed higher effect on the biodegradation than the single strains. Aeration found to be the most important in the effect on the biodegradation results.

ABSTRAK

Sebanyak Lima sampel tanah tercemar dengan benzene, minyak kereta terpakai dan diesel diambil daripada lima bengkel kereta di sekitar Kuala Lumpur, Malaysia untuk tujuan kajian ini. Bagi tujuan itu, strain mikrob diasingkan dengan menggunakan medium terpilih (agar yang mengandungi minyak mentah, minyak terpakai kereta dan benzene).Ujian biokimia juga dijalankan untuk mengenalpasti mikroorganisma yang ada dan digunakan dalam eksperimen biodegradasi dua jenis minyak mentah (Miri dan Masila) dan minyak terpakai kereta. Penentuan secara kualitatif kapasiti degradasi minyak mentah dan minyak terpakai kereta dijalankan dalam 24 bekas kluster kultur sel – yang mempunyai dasar yang rata. Brot nutrient medium , minyak mentah dan minyak terpakai kereta dimasukkan ke dalam setiap bekas. Kultur mikroorganisma ini kemudiannya diasingkan dalam tiub (tunggal dan bercampur) dan diinkubasi pada suhu 27°C selama 30 hari. Bagi mengukur kadar hidrokarbon biodegradasi, tanah yang telah disteril dimasukkan kedalam piring petri dan ditambah dengan 3% w/w minyak mentah dan minyak terpakai kereta secara berasingan dan kemudiannya ditambah dengan strain yang diasing (tunggal dan bercampur). Salah satu daripada piring petri tersebut digunakan sebagai piring kawalan tanpa menambah apa-apa mikrob. Daya serap minyak mentah ditentukan dengan spektrofotometer pada 360nm dan untuk minyak terpakai kereta pada 340nm. Kajian juga dibuat untuk melihat kesan pengudaraan, penambahan NPK dan penambahan mikrob pengdegradasi pada biodegradasi minyak mentah Masila dan minyak terpakai kereta. Tanah tersebut telah ditambah dengan 20% minyak mentah Masila dan minyak terpakai kereta secara berasingan.Tanah tersebut kemudian dibahagikan kepada beberapa bekas. Bekas (1) diudarakan dua kali seminggu, NPK bernisbah 1:1:1 pula dimasukkan kedalam bekas (2), kesemua campuran strain yang diasingkan dan mempunyai kapasiti degradasi dimasukkan kedalam bekas (3). Disamping campuran mikrob, NPK yang diudarakan secara berterusan dimasukkan kedalam bekas (4). Satu bekas pula digunakan sebagai bekas kawalan tanpa menambah bahan lain(tanah dan bahan cemar sahaja).Unit Pembentukan Koloni (Colony Forming Unit) keseluruhan mikrob heterotropik dan mikrob menggunakan hidrokarbon, PH dan peratus degradasi minyak juga ditentukan.Sebanyak 22 strain mikrob diasingkan dan dikenalpasti sebagai *Achrombacter*, *Aeromonas*, *Klebsiella pneumonia*, *pseudomonas*, *Corynebacterium*, *Penicillium*, *Enterobacteriaceae (Enteric rods)*, *Actinobacillus*, *Streptomyces*, budding yeast cells, *Cladosporum dan Geotrichnum* spp. Selepas 30 hari keputusan biodegradasi tertinggi bagi minyak mentah Miri ialah 54.33% dan 84.61% untuk strain Z13 (*corynebacterium* spp*)* dan strain campuran mikrob yang diasingkan dari Rawang dan Serdang secara berturut. Sementara itu peratusan untuk strain A3 (*Klebsiella pneumonia*) untuk minyak mentah Masila ialah 33.81% dan 49.47% untuk strain mikrob campuran yang diasingkan dari Serdang (Smix).Dalam eksperimen biodegradasi minyak kereta, strain Z4 (*Corynebacterium*) menunjukkan peratus degradasi tertinggi iaitu 72.9%. Sementara itu campuran mikrob strain yang diasingkan dari Kajang (Zmix) menunjukkan 72.4% degradasi. Dalam eksperimen yang mengkaji kesan pengudaraan dan penambahan NPK keatas biodegradasi menunjukkan bahawa bekas yang diudarakan mengalami degradasi sebanyak 56.62% selepas 42 hari di dalam minyak mentah Masila. Sementara itu bekas yang mengandungi NPK yang diudarakan dan ditambah dengan strain yang diasingkan menunjukkan degradasi sebanyak 66% dalam minyak kereta. Secara amnya, dua spesis bakteria dan satu spesis fungus terasing didapati berkesan sebagai pengdegradasi (*Corynebacteruim* spp, *Streptomyces* spp. Dan *Cladosporum* spp). Degradasi tertinggi oleh strain tunggal ialah pada minyak terpakai kereta mungkin disebabkan oleh kebolehan mengadaptasi mikrob terasing yang digunakan. Campuran mikrob menunjukkan kesan yang lebih tinggi keatas biodegradasi berbanding strain tunggal. Pengudaraan merupakan sesuatu yang penting dalam memberi kesan kepada keputusan biodegradasi.

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Ca	Calcium
CO_2	Carbon dioxide
°C	Celsius
cm	Centimeter
g	Gram
hr	Hour
Kg	Kilogram
Km	Kilometer
Pb	Lead
L	Liter
$<$	Lower than
Mg	Magnesium
m	Meter
µg	Microgram
µL	Microliter
µL/L	Microliter per liter
µm	Micrometer
mg	Milligram
mg/L	Milligram per liter
mL	Milliliter
mm	Millimeter
min	Minute
nm	Nanometer

ppm	Part per million
%	Percentage
pH	Potential hydrogen
sec	Second
v/v	Volume per volume
w/w	Weight per weight
Zn	Zinc

LIST OF ABBREVIATIONS

NH_4NO_3	Ammonium nitrate
$(NH_4)_2SO_4$	Ammonium sulfate
ANOVA	Analysis of variance
BTEX	Benzene, toluene, ethylbenzene, and the xylenes
C	Carbon
CA	Constant aeration
DNA	Deoxyribonucleic acid
Na_2HPO_4	Disodium hydrogen phosphate
dH_2O	Distilled water
FID	Flame ionization detector
GC	Gas chromatography
GCms	Gas chromatography-mass spectrometry
G -ve	Gram negative
G +ve	Gram positive
HMW	High molecular weight
HMW PAH	High molecular weight polycyclic aromatic hydrocarbons
IA	Intermittent aeration
$MgSO_4$	Magnesium sulfate
MSB	Minimal salts basal
mix	Mixture
NPK	Nitrogen, phosphate and potassium
N/P	Nitrogen phosphate ratio
NAPL	Nonaqueous phase liquid

PHCs	Petroleum hydrocarbon contents
PCBs	Polychlorinated biphenyls
PAHs	Polycyclic aromatic hydrocarbons
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
rRNA	Ribosomal ribonucleic acid
NaCl	Sodium chloride
SDS	Sodium dodecyl sulphate
Spp.	Species
SD	Standard deviation
SPSS	Statistically backage for social sciences
TPH	Total petroleum hydrocarbon
TCE	Trichloroethylene
UV	Ultraviolet
UK	United kingdom
US	United states
USA	United states of America
UKM	Universiti kebangsaan malaysia
UMP	Universiti Malaysia Pahang
UPM	Universiti Putra Malaysia
WA	Without aeration

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

The quality of life on the planet depends on the quality of the environment. Years ago, we were sure that we had many land and resources; today, however, the real picture shows, in greater or lesser degree, our negligence and carelessness in using them. In many countries the problems associated with contaminated sites became more serious. Contaminated sites in general resulted from previous industrial activities, when the connection between production, use, and disposal of hazardous substances with health and environmental effects were less well recognized than today. The problem is global, and the estimated number of contaminated sites is significant (Cairney, 1993). The awareness of potential threat to human health because of contaminated land is now much bigger, and its continual discovery over recent years has led to international efforts to remedy many of these sites, either as a response to the risk of adverse health or environmental effects caused by contamination or to enable the site to be redeveloped for use. (Vidali, 2001).

Fossil fuels represent primary energy source in the global industry. Due to fossil fuel manufacturing and imports/exports, there is a big threat on environmental pollution, and serious ecological damage. This is the result of fuel by-products and spills in sites where storage, transport, distribution, refining, consumption and the existing industries related fossil fuel can cause harm. The interest of scientists to investigate the oil distribution and its fate in the environment increases because of this fact, especially in the marine environment. About five million tons of crude oil and refined oil go into the ecosystem every year due to anthropogenic sources from oil spills (Hinchee and Kitte, 1995). Analysis of reported oil spills shows that most of the oil comes from tankers, barges and other vessels as well from land pipeline spills. Extensive changes in marine, in addition to terrestrial ecosystems resulting from the grounding of the Exxon Valdez (1989), the Nahodka oil spill, the Erica spill (1999) and the Prestige spill (2002), have increased the attention of biotechnologists, chemists, environmentalists, and engineers (Braddock et al., 1995; Tazaki et al., 2004).

Used engine oil is one of the sources for soil pollution with hydrocarbons. Used engine oil contains petroleum ether, benzene, gasoline, naphthalene, mineral spirits, kerosene, and fuel oil, paraffin wax, lubricating oil, tar or asphalt. Used car oil has much higher concentrations of PAHs (polycyclic aromatic hydrocarbons) compared with new motor oil (Irwin et al., 1997).

Traditional remediation methods include containment material and physical removal. These methods also use chemicals, especially shoreline cleaners, which are usually organic solvents with or without surfactants (Riser-Roberts, 1992). The cleaning of shoreline with surfactants emulsifies the adsorbed oil, which can entrain adjacent waters or is even transported deeper into the soil of the shoreline. The oil solvent mixtures are collected by conventional skimming methods. Mechanical recovery of oil includes oil sorbents. Sorbents can transform oil to a transportable form for short-term storage. Almost all the physicochemical methods use chemical agents, in addition to their emulsion with oil, which can cause toxicity, to aquatic organisms. They represent another source of pollution and increase the cost of the oil recovery. Additionally, abiotic losses because of evaporation of low molecular hydrocarbons, dispersion and photooxidation (for aromatic compounds only) play an important role in decontamination of the oil spill environments (Mills et al., 2003).

There is an increased interest to promote environmental methods in the process of cleaning oil-polluted sites. These methods cost less and do not introduce chemicals to the environment. In comparison with physiochemical methods, bioremediation is a very feasible alternative for an oil spill response. This technique is considered an effective technology for treatment of oil pollution. One reason is that the majority of the molecules in the crude oil and refined products are biodegradable (Malatova, 2005).

1.2 PROBLEM STATEMENT

Because of the rapid industrialization processes and the big worldwide energy demands, oil spills become a global problem, especially in industrialized countries. International Oil Spill Database shows that since the early 1960s, nearly 1135 million litres of oil have spilled into US marine waters which occurred in 826 incidents involving tankers, barges, and other vessels, and about 757 million litres of oil have spilled onto US soil from the land pipeline spills (on average, 99 land pipeline spills per year). An estimated 1892 million and over 757 million litres of oil have spilled from tankers in Europe and Asia Pacific since 1965, respectively. The high quantities of spilled oils and petroleum products go into our sea, territory as well as groundwater, which cause severe damage to marine life, terrestrial life, human health, and natural resources (Wang et al., 1999). One example of the most recent oil spill accident is the Deep water Horizon oil spill or the BP oil spill in the Gulf of Mexico which happened in 2010. It is the largest accidental marine oil spill in the history of the petroleum industry. About 4.9 million barrels of crude oil was released into the sea (Rick and Alan, 2010).

In Asia during the last few years, there were major oil spill accidents at different places of the country in January 21/1993 in Singapore, Indonesia, and Malaysia**.** Two million barrels of oil was leak and burned, and the leak spread a slick approximately 35 miles (56 km) along Sumatra drifting towards India's Nicobar Islands. In September 2/2000 Malaysia, 116 Tons of diesel oil spilled after a cargo sank due to a collision. In May 28/2001 Malaysia, an oil tanker with 67 tons of diesel fuel, and 1,500 Tones of bitumen, sank after a crash from behind by a super tanker. Diesel and bitumen started to spill into the sea, and spread to about one nautical mile from the spot of collision. In June 13/2001 Malaysia, an Indonesian tanker loaded with a toxic chemical capsized off Malaysia's southern state of Johor, 18 Tons of diesel, and 600 Tons of the poisonous industrial chemical phenol were spilled. The toxic spill killed thousands of marine creatures, and Singapore authorities have also warned its citizens to stay away from nearby waters (The Marine Group).

Engine oil is the most worldwide used petroleum product. The inevitable leakage and abandoned remnant of engine oil that is used as lubricant pollute the environment

(Liang et al., 2007). Only 1 gallon of used motor oil is enough to contaminate 1000 000 gallons of freshwater. Used engine oil also represents potential threat to humans, animals, and vegetation (Edewor et al., 2004). The most significant and largest proportion of oil spill is from internal combustion engines (petrol and diesel engines) in road transport and shipping (Porst, 2000). The components in used motor oil cause harmful effects on liver, kidneys, heart, lungs and nervous system (Irwin et al., 1997). One of the most significant impacts associated with workshop seepage of used motor oil includes loss of soil fertility, holding capacity of water, permeability and binding capacity (Khan and Rizvi, 2011).

Interest in the microbial biodegradation of pollutants was intensified recently to find sustainable ways to remediate contaminated environments. Bioremediation and biotransformation are methods, which use the naturally occurring ability of microbial metabolism to degrade, transform or accumulate a huge range of compounds including hydrocarbons such as crude oil and its different products (Alexander, 1999).

1.3 OBJECTIVES

The primary objectives of this study are:

(i) To isolate and identify hydrocarbon degrading microorganisms from contaminated soil in Malaysia by selective enrichment techniques.

(ii) To determine the rate of biodegradation of two types of crude oil (Miri and Masila) and used car oil by isolated microorganisms

(iii) To study the ability of single strains in comparison with microbial blends to degrade petroleum and used car oil.

(iv) To study the effect of aeration, NPK and microbial degraders, which have been added to Masila crude oil and used car oil biodegradation.

1.4 SCOPES OF STUDY

- Collection of contaminated soil samples from various car workshop areas in Kuala Lumpur region (Kuala Lumpur, Selangor, Kajang, Rawang and Serdang), which are known to be contaminated with different types of hydrocarbons such as benzene and used engine oil.

- Microorganism's isolation by using selective mediums (agars containing crude oil, used car oil and benzene).

- Identification of isolated microorganisms by using simple Gram staining technique and biochemical tests to determine the type of the strain which was effective as a hydrocarbon degrader.

- Determination of the rate of biodegradation of three types of hydrocarbons (used car oil, Miri and Masila crude oil) quantitatively, using spectroscopic techniques to determine microbial efficacy as hydrocarbon degrader.

- Finally, study of the effect of aeration, NPK and microbial degraders adding on used car oil and Masila crude oil biodegradation using spectroscopic technique.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Contamination of soil and water with hydrocarbons poses a major ecological and human health problem that needs an effective and affordable technological intervention. Many sites stay contaminated with no treatment in sight because it is very expensive to clean them with the available technologies. Bioremediation can provide an economic solution for remediating many of these sites (Martello, 1991).

Bioremediation is the application of biological treatment to the cleanup of harmful chemicals. This process includes detoxification, where the waste is converted to less toxic compounds, and mineralization, which is a process of transforming the waste materials into inorganic compounds such as carbon dioxide, water and methane (Gavrilescu, 2010; Martello, 1991). Bioremediation is one environmental biotechnology technique. For many years, microorganisms have been used to eliminate organic matter and toxic chemicals from domestic and industrial waste effluents. However, the range of treatments has been developed greatly due to the great improvement in available biotechnology. Bioremediation is now the technology of choice for the remediation of many polluted environments, especially areas contaminated with petroleum hydrocarbons. Recently, serious efforts have been made to use nature's biodegradative ability aiming at large-scale technological applications for effective and economic environmental restoration (MABIC, 2005).

The regular techniques used for remediation depend on digging up contaminated soil and removing it to a landfill, or capping and containing the contaminated areas of a site. The methods have some disadvantages. The first method simply transfers the contamination somewhere else and may make major risks in the drilling, handling, and transporting of hazardous material. Moreover, it is very difficult and expensive to find new landfill sites for disposal of the material. The cap and contain method is only a temporary solution because the contamination remains on site, requiring observation and maintenance of the isolation barriers long into the future, with all the associated costs and potential responsibility (Vidali, 2001).

A better way than these conventional methods is to completely destroy the contaminants if possible, or transform them to less harmful substances. High-temperature incineration and various types of chemical decomposition (e.g., base-catalyzed dechlorination, UV oxidation) are the technologies that have been used. They can be effective at minimizing levels of any contaminants, but they have several side effects. Generally, the technology is very complex; the cost for small-scale application is high, and the difficulty of being accepted by public, particularly for incineration, which can increase the exposure to contaminants for workers at the site and residents living nearby (Vidali, 2001).

Bioremediation offers the possibility of destroying or rendering harmless various contaminants by using naturally occurring biological activity. It uses relatively cheap, simple technology techniques, which mostly have a high public acceptance and may often be carried out on site. It cannot be suitable all the time;, however, if the range of pollutions on which it is effective is limited, the time scales involved are prolonged, and the levels of residual contaminant that can be achieved may not always be suitable. Though the methods employed are not complex, large experience and expertise will be needed to design and apply a good bioremediation program, because of the need to thoroughly assess a site for suitability and to optimize conditions to fulfill a satisfactory result (Vidali, 2001).

Bioremediation has been used at many sites over the world, including Europe, with different degrees of achievement. Techniques are being developed as more experience and knowledge are gained, and bioremediation has great potential for treating certain types of site contamination. Unfortunately, the principles, techniques, advantages, and disadvantages of bioremediation are not widely known or understood, especially among those who will have to deal directly with bioremediation proposals, such as site owners and regulators. Here, we intend to assist by providing a straightforward, pragmatic view of the processes involved in bioremediation, the pros and cons of the technique, and the issues to be considered when dealing with a proposal for bioremediation. Some tests make an exhaustive examination of the literature of bioremediation of organic (Norris et al., 1993) and inorganic pollutants (Hinchee and Kitte, 1995), and another test takes a look at pertinent field application case histories (Flathman et al., 1993).

Polycyclic aromatic hydrocarbons (PAHs) are very important pollutants found in soil, air and sediments. These contaminants are introduced into the environment through various methods. PAHs products and their derivatives are widely distributed due to incomplete combustion of organic substances arising, in apart from natural combustion as in volcanic eruptions and forest fires, but the most important reason is human intervention such as industries and the related accidents. In recent years, industrial production, transportation, storage, refining, consumption and distribution are the major causes of PAHs contamination. The fate of polycyclic aromatic hydrocarbons in environment is of great concern due to their toxic, mutagenic, and carcinogenic effects (Fawell and Hunt, 1988; Mas et al., 2010). PAHs can permeate into organic-rich soils and sediments, can be accumulated in marine living creatures, and then transferred to humans through seafood consumption (Meador et al., 1995).

2.2 BIOREMEDATION

As a definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses natural bacteria and fungi or plants to degrade or detoxify substances harmful to health of human and/or the environment (De Wilde et al., 2007; Vidali, 2001). By other means, bioremediation is the intentional use of biological degradation procedures to remove or reduce the concentration of environmental pollutants from sites where they have been released. The concentrations of pollutants are reduced to levels considered acceptable to site owners and/or regulatory agencies (Figure 2.1). The microorganisms could be indigenous to contaminated sites or they may be isolated from somewhere else and introduced to the contaminated area. Pollutant compounds are transformed by organisms through reactions that happen as part of the metabolic processes. Compound biodegradation is normally a result of the actions of a group of organisms (Vidali, 2001).

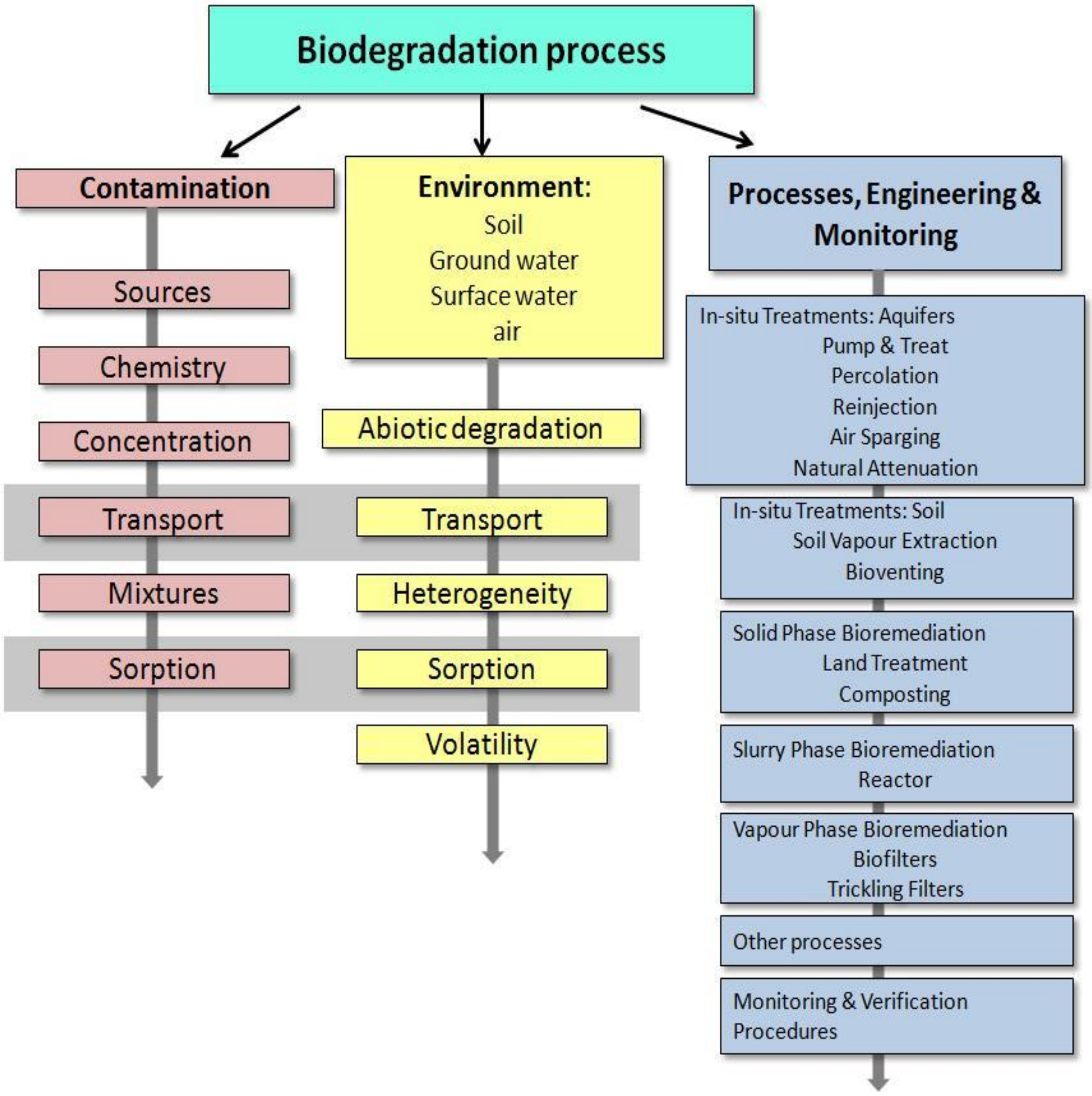

Figure 2.1 Overview of Bioremediation Process

Source: Santra (2010)

2.2.1 History of Bioremediation

Bioremediation is not a new concept;, scientists have discovered the process since 1940s. Bioremediation was made known to the public in the U.S. only in 1980s as a procedure of removing contaminants from coastal area polluted with oil. The *Exxon Valdez* oil spill in 1989 in Prince William Sound, Alaska was the first step for this concern. Since 1989, bioremediation has been involved, discussed, and applied in many circumstances (Hoff, 1993).

The bioremediation evolution as an oil spill removing technology gives a good example of how a new ecological technology evolves. The history of bioremediation in spill response is divided into three periods (Hoff, 1993);, 'courtship' period, which is prior to 1989, the 'honeymoon' period, which is from 1989 until 1991 and the 'establishment' period, which is after 1992. The courtship period (Pre-1989) was a research period, when bioremediation was not well known outside the microbiology or hazardous waste community. Numerous published articles from 1970s and earlier reported the process of microbial degradation of oil, both in the laboratory and in field experiments. Some scientific papers on this issue were published during the 1970s and 1980s, as well as many review papers covering mechanisms of biodegradation, and papers submitting results from controlled field trials measuring rate of degradation in different environments. Many studies, which followed large oil spills like the *Amoco Cadiz*, measured degradation of oil in the environment and confirmed earlier published results from laboratory studies (Hoff, 1993). During the honeymoon period (1989 to 1991), bioremediation broadened attention and interest. The end of this period was disappointing as the promise of the technology was not always fruitful by its application in real field. This period ended in 1990 and 1991, and as the result, monitoring of the bioremediation applications became available. None of the studies conducted outside Alaska could confirm the effectiveness of bioremediation applications in field tests. Many of these tests had poor design, were conducted over very short a time period, or had analytical difficulties measuring changes in oil concentrations (Hoff, 1993). The establishment period started in 1992. During this period, bioremediation has achieved a certain level of acceptance, with more realistic expectations than before, but the interest and attention level has decreased considerably. The doubt about the toxicity of several